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Effect of Leptin on Mouse Trophoblast Giant Cells[1]

L.C. Schulz,[2,3,5] E.P. Widmaier,[5] J. Qiu,[4] and R.M. Roberts[3]

Division of Animal Sciences[3] and Department of Statistics,[4] University of Missouri, Columbia, Missouri
Department of Biology,[5] Boston University, Boston, Massachusetts

ABSTRACT

Leptin plays a role in both energy homeostasis and reproduction, and it is required in early pregnancy. It stimulates metalloproteinase activity in cultured human trophoblasts and invasiveness of cultured mouse trophoblasts. Our goal has been to examine mechanisms that underpin the ability of leptin to promote trophoblast invasiveness in primary cultures of mouse trophoblasts. Leptin stimulated the phosphorylation of MEK (MAP2K1) but not signal transducer and activator of transcription 3 (STAT3) in the cultures, increased the concentration of the suppressor of cytokine signaling 3 (SOCS3) protein, and upregulated metalloproteinase activity. Microarray analysis revealed that leptin stimulated select genes with roles in cell motility, including *Stmn,* a gene linked to invasiveness in other cell types. There was also an increase in activity of several genes associated with MAPK and RhoGTPase signaling. In addition, leptin muted expression of genes correlated with terminal differentiation of trophoblast giant cells, including ones associated with the TGFbeta signaling pathway and endoreduplication of DNA, and upregulated selected prolactin-related family members. Feulgen staining of leptin-treated cells revealed a loss of cells with low ploidy. The data suggest that leptin accelerates disappearance of non-giant cells while inhibiting terminal differentiation of committed giant cells, possibly by maintaining cells in an intermediate stage of differentiation.

leptin, matrix metalloproteinase, MEK, placenta, signal transduction, SOCS3, STAT3, trophoblast

INTRODUCTION

Although first discovered as a regulator of energy homeostasis, the hormone leptin is also important for reproduction. The *Lep*^{ob} (also known as *ob*/*ob*) mouse, which lacks functional leptin protein, is infertile. Female *Lep*^{ob} mice can only bear young if given replacement leptin by means of injections through Day 6.5 postcoitum, a time well after the initial invasion of trophoblast cells into the endometrium and roughly coinciding with the development of the ectoplacental cone and formation of a rudimentary placenta [1, 2]. In addition, intrauterine injections of a leptin antagonist block implantation [3]. The placenta has been shown to express both long and short isoforms of leptin receptors throughout pregnancy in multiple species, including humans and mice [4–7]. Leptin actions in placental trophoblast cells include stimulation of human chorionic gonadotropin and interleukin 6 production, and inhibition of progesterone production [8, 9]. Leptin has also been shown to stimulate the release of matrix metalloproteinase 2 and the activity of matrix metalloproteinase 9, enzymes involved in trophoblast invasion, by cultured human trophoblast cells [10]. We have previously shown that leptin stimulates invasion of cultured trophoblast cells through a Matrigel-coated membrane and that this activity is dependent on metalloproteinase activity. The goal of the present study is to identify intracellular signaling pathways that mediate these effects of leptin and, using RNA profiling, determine how leptin influences the phenotype of the invasive cells that are targeted.

Leptin actions at the intracellular level have been studied extensively in the hypothalamus, where it activates multiple signaling pathways, including ones involving JAK2/signal transducer and activator of transcription 3 (JAK2/STAT3), MEK/ERK, PI3 kinase, erbB2, and IRS1. Only the longest leptin receptor isoform, LEPRb, is capable of signaling through the STAT3 pathway. Both the long and the short (LEPRa) receptors can activate the MEK/ERK pathway, although the latter can signal only weakly compared with the long form [11]. Mutation of the STAT3-activating residue of the leptin receptor (Y1138) results in impairments in appetite and metabolism that are nearly as severe as those observed in the *db*/*db* mouse, which has only a truncated form of LEPRb [12]. However, unlike the *db*/*db* mouse, the Y1138 mouse is partially fertile, which suggests that STAT3 is not as important for leptin regulation of reproduction and, specifically, trophoblast invasion as it is for regulation of energy homeostasis [12].

Both the STAT3 and MEK/ERK signal transduction pathways have been implicated in the abilities of other factors to stimulate metalloproteinase activity and trophoblast invasive properties. For example, leukemia inhibitory factor (LIF) [13] exerts its effects via STAT3, whereas several other growth factors act via MEK/ERK [14–16]. Thus, both signaling pathways are candidates for leptin stimulation of trophoblast invasion. However, even at superphysiological levels, leptin fails to turn on STAT3 signaling in the transformed trophoblast cell line BeWo [17]. Instead, both BeWo and JAr cells activate their MEK/ERK pathways upon leptin treatment [17, 18]. Importantly, MEK mediates leptin-stimulated cell proliferation in these cell lines, an effect not observed in primary mouse or bat trophoblast cells [19, 20]. Because the function of leptin is divergent among these cell types, it is difficult to predict whether the signaling pathways will be conserved. Here, we examined activation of the STAT3 and MEK/ERK pathways by leptin in primary mouse trophoblast cells to test the hypothesis that at least one of these pathways would be required for leptin stimulation of matrix metalloproteinase activity. We also hypothesized that leptin would have broader effects on trophoblast invasion, perhaps by influencing the differentiation of trophoblast cells toward the invasive subtype and/or influencing the invasive behavior of those cells.

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[2]Correspondence: Laura Schulz, 240h Bond Life Sciences Center, 1201 E. Rollins St., Columbia, MO 65211. FAX: 573 884 9394;
e-mail: schulzl@missouri.edu

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Accordingly, we employed microarray analysis to identify changes in gene expression that occur as a result of exposure to leptin over time.

MATERIALS AND METHODS

Chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Animals

All animal procedures were approved by the Boston University or University of Missouri–Columbia institutional animal care and use committees and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Timed-bred, Swiss-Webster mice were obtained from Taconic (Germantown, NY) or Harlan (Indianapolis, IN). The day that a vaginal plug was detected was considered Gestation Day 0. On Day 10 of pregnancy, mice were killed by CO_2 inhalation, and placentas were collected for trophoblast cell culture.

Trophoblast Cell Cultures

Trophoblast cells were cultured according to a previously described method [21], with modification [20, 22]. Briefly, placentas were separated from the underlying endometrium using dissecting forceps in a sterile dish containing wash medium (Medium 199; 20 mM Hepes, 10 mM NaHCO3, and penicillin/streptomycin). The placentas then were incubated in dissociation medium (wash medium containing 1 mg/ml collagenase and 20 μg/ml DNase) for up to 1 h at 37°C, with periodic pipetting to separate cells. Cells were washed to remove dissociation medium, then collected and filtered to remove undigested tissue. The cells were separated on an isotonic 40% Percoll gradient at 30 000 × *g* for 30 min. The trophoblast cell layer was transferred to a new tube, washed in wash buffer, and plated in NCTC-135 medium, with 20 mM Hepes, 10 mM NaHCO3, 1.65 mM cysteine, 10% fetal calf serum, and penicillin/streptomycin on Matrigel-coated plastic wells. Cells were incubated in this medium for 14–18 h for optimal adherence before experiments were begun.

For Western blots and ELISAs, cells were exposed to serum-free NCTC-135 medium for 24 h to avoid potential exposure to leptin. Then, cells were treated with 50 ng/ml leptin for 0, 3, 10, 30, or 60 min. This concentration was chosen because it was the most effective at stimulating trophoblast invasion in vitro in a previous study [20] and is roughly equal to the peak concentration of leptin during pregnancy in mice [23]. At the end of each treatment period, cells were washed briefly in sterile PBS and collected by scraping in a commercial lysis buffer (Cell Signaling Technology, Danvers, MA) with protease inhibitor cocktail (Sigma) added.

For the determination of metalloproteinase activity, cells were cultured in a total of eight different conditions, either in the presence (even numbers) or absence (odd numbers) of 50 ng/ml mouse recombinant leptin: (1 and 2) serum-free medium (control) or serum-free medium containing (3 and 4) 0.04% dimethyl sulfoxide (DMSO), (5 and 6) 10 μM MEK inhibitor U0126 (Calbiochem, San Diego, CA) dissolved in DMSO, or (7 and 8) 2 μM STAT3 inhibitor cucurbitacin I (Calbiochem) dissolved in DMSO. After 24 h of treatment, medium was collected and immediately used for metalloproteinase assays or aliquoted and frozen at −80°C.

Western Blotting

Lysates were centrifuged at 1000 × *g* for 5 min to remove insoluble materials. Lysates were boiled in Laemmli sample buffer containing 10% 2-mercaptoethanol [24]. Twenty microliters of each sample was separated by electrophoresis on a 10% polyacrylamide gel, and the proteins were transferred to a polyvinylidene fluoride membrane. Membranes were incubated in 4% bovine serum albumin (BSA) for 1 h to block nonspecific binding. The blots were incubated overnight at 4°C in primary antibody in 2 ml of 2% BSA. Antibodies to STAT3 and phosphoserine 727 STAT3 were obtained from Cell Signaling Technology and were each used at a 1:300 dilution. Anti-suppressor of cytokine signaling 3 (anti-SOCS3) antibody (AbCam, Cambridge, MA) was used at a 1:100 dilution. After three 10-min washes in Tris-buffered saline, the blots were exposed to anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Cell Signaling Technology) at a 1:500 dilution in 2% BSA for 1–2 h. After washing, blots were incubated with chemiluminescent peroxidase substrate (Cell Signaling Technology), and bands were imaged with a Fuji LAS 3000 Imaging System (Fujifilm Medical Systems USA).

Blots were stripped in 0.2 M NaOH for 30 min and then blocked and reprobed with an antibody to β-actin (Cell Signaling Technology) at a 1:500 dilution, followed by secondary antibody incubation and chemiluminsecent detection as described above. Band densities were quantified using Image J software (National Institutes of Health, Bethesda, MD). Data were analyzed by repeated-measures ANOVA with Bonferroni pairwise or Dunnett multiple (Pser-STAT3) comparison tests by employing GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego, CA).

ELISAs

Commercial ELISA kits were used to quantify phospho-tyrosine-705-STAT3, phospho-MEK 1/2, and total MEK 1/2 (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, 100 μl cell lysate was added to each well, and the plate was incubated overnight at 4°C. Plates were washed with the included wash buffer using a squirt bottle. Detection antibody solution was added to each well, and the plate was incubated for 1 h at 37°C. Plates were washed and then incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min at 37°C and washed again. After incubation with peroxidase substrate, plates were read at 450 nm on a plate spectrophotometer (BioTek). Data were analyzed as described for Western blot data above.

Metalloproteinase Activity Assay

Metalloproteinase activity was quantified in culture medium by the EnzChek Gelatinase/Collagenase Assay Kit (Molecular Probes) according to manufacturer's recommendations. Samples of each medium (100 μl) were incubated with 20 μg fluorescein-conjugated gelatin in 1× reaction buffer for 48–120 h until sample readings were above background levels. Enzyme activity was determined by measuring fluorescence intensity at 528 nm in a fluorescence plate reader (BioTek) and subtracting fluorescence derived from culture medium only. Collagenase type IV from *Clostridium histolyticum* (Molecular Probes) was used as a positive control. Data were analyzed by one-way repeated-measures ANOVA followed by Tukey test by using GraphPad Prism.

Zymography

Medium was concentrated with a Microcon filter (Millipore, Bedford, MA) with a nominal molecular mass cutoff of 30 kDa. Concentrated samples were analyzed by SDS-PAGE under nondenaturing conditions on a 10% gel containing 1% porcine skin gelatin [25]. Gels were washed three times for 15–20 min each in 2.5% Triton-X 100 to remove SDS. They then were incubated overnight at 37°C in collagenase activation buffer (50 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.5). After a brief rinse in distilled water, gels were stained with Gelcode blue [26] for 1 h, and then destained in distilled water for 15 min before image capture. Clear bands against a blue-stained background revealed gelatinase activity. Colors were inverted using Photoshop CS2 (Adobe, San Jose, CA) to provide a better contrast for the bands.

Microarray Analysis

Illumina microarrays containing 24 886 probe sets were used to measure changes in gene expression as a result of leptin treatment in the mouse primary trophoblast cells. To identify the cascade of events occurring over time, expression was analyzed after 1, 6, and 24 h of leptin treatment. Cells cultured in the absence of leptin also were examined at 1 and 24 h. Four replicate experiments (nos. 15, 19, 27, and 28) were performed. For each experiment, placentas from five to eight Day 10 pregnant mice were pooled for trophoblast isolation. The trophoblast cell pool was distributed equally into five wells on a Matrigel-coated, six-well plate in medium containing 10% fetal bovine serum as described above. One well was used for each treatment group. After overnight culture, cells were exposed to either control serum-free medium (CON) or medium with 50 ng/ml recombinant mouse leptin (LEP). Cells then were collected by scraping in TRI Reagent at 1 h (CON and LEP), 6 h (LEP), and 24 h (CON and LEP) after treatment. After phase separation, the aqueous phase was removed and mixed with an equal volume of ethanol. This mixture was applied to an RNEasy column (Qiagen, Valencia, CA) and RNA purified according to the manufacturer's instructions.

Microarray analysis was conducted by the University of Missouri DNA Core Facility. Biotin-labeled antisense RNA (aRNA) target was made from 0.5 μg total RNA by using the Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX) according to the manufacturer's procedures. Briefly, the total RNA was reverse transcribed to first-strand cDNA with an a oligo(dT) primer bearing a 5′-T7 promoter by using ArrayScript (Ambion) reverse transcriptase. The first-strand cDNA then underwent second-strand synthesis and cleanup to become the template for in vitro transcription. The biotin-labeled aRNA was synthesized using T7 RNA polymerase with biotin-

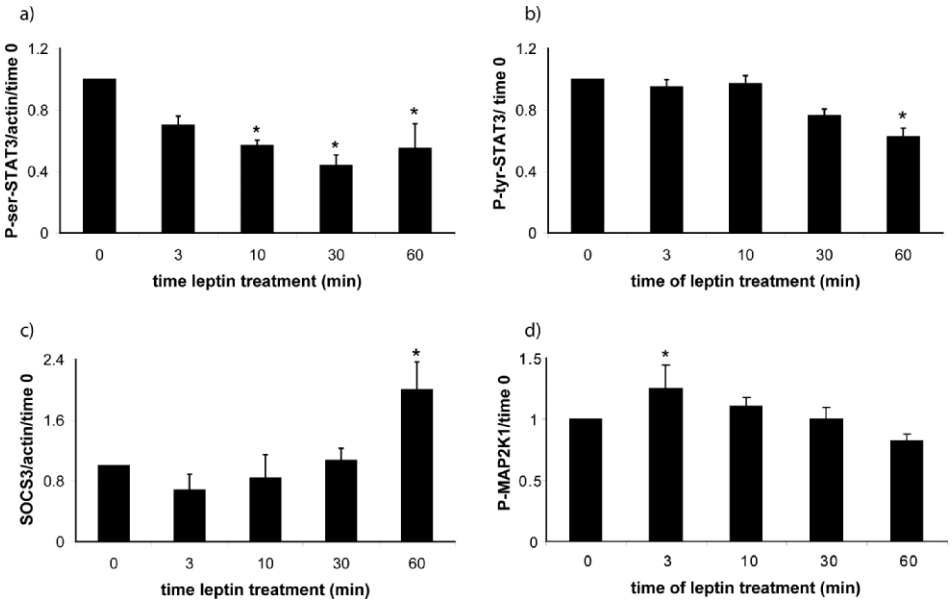

FIG. 1. Primary mouse trophoblast cells were treated with 50 ng/ml leptin and lysed at 3, 10, 30, and 60 min after treatment. **a**) Effect of leptin on phosphorylation of STAT3 at serine 727, as measured by Western blotting. **b**) Effect of leptin on phosphorylation of STAT3 at tyrosine 705, as measured by ELISA. **c**) Effect of leptin on SOCS3 expression, as measured by Western blotting. **d**) Effect of leptin on phosphorylation of MAP2K1 (MEK) at serines 217/221, as measured by ELISA. Protein concentrations are shown relative to those at time 0 for each experiment. Band densities were normalized to actin band densities to control for loading (**a** and **c**). P-MAP2K1 (MEK) was normalized to total MAP2K1 (MEK), also measured by ELISA (**d**). The protein of interest changed significantly over time in all four experiments (repeated-measures ANOVA; $P = 0.05$). *Significant difference from time 0 (**a**: Dunnett $P < 0.05$; **b**–**d**; Bonferroni $P < 0.05$). Bars represent standard error. N = 3 (**a** and **c**) and N = 7 (**b** and **d**).

nucleotide triphosphate mix and purified. The aRNA was hybridized to Illumina Sentrix MouseRef-8 Expression BeadChip arrays (Illumina, San Diego, CA) at 58°C for 20 h. Three bead chips, each of which contained eight arrays, were used for the experiment. RNAs from the CON 1 h, LEP 1 h, LEP 6 h, and LEP 24 h of experiment 19 were hybridized on the first chip. The same treatment groups from experiments 27 and 28 were hybridized on the second chip. Those treatment groups from experiment 15 plus all of the CON 24 h samples were hybridized on the third chip. After hybridization, the chips were washed and stained with streptavidin-C3. The image data were acquired by a BeadArray reader (Illumina, San Diego, CA).

Microarray Statistics

Summary intensities of 24 886 probe sets on 19 arrays of three BeadChips (excluding one outlier array—experiment 28, control 24 h—which was removed from the statistical analysis) were obtained using Illumina's BeadStudio and then quantile normalized using beadarray software [27]. Four paired *t*-tests were applied to the base 2 logarithm-transformed normalized data by means of limma software [28] to identify differentially expressed genes 1) between control and leptin-treated samples at 1 h, 2) between leptin treatments of 1 h and 6 h, 3) between control and leptin-treated samples at 24 h, and 4) to identify genes that have differential profile patterns over time in the leptin-treated samples versus the control samples ([24 h − 1 h leptin] − [24 h − 1 h control]).

Real-Time RT-PCR

Selected microarray results were confirmed by real-time RT-PCR. Trophoblast cells were treated for 24 h with or without 50 ng/ml leptin and then lysed for RNA collection as described above for the microarray analysis. Three independent pairs of samples (CON and LEP) were used. Two of the pairs were used previously in the microarray analysis. The third was obtained by pooling RNA from two independent trophoblast cell experiments that had been performed in the same way. Genomic DNA was removed using Turbo DNase (Ambion) according to the manufacturer's protocol. The RNA was then reverse transcribed with SuperScript III (Invitrogen, Carlsbad, CA). Commercially designed primer/probe mixes were obtained for *Mdh2*, *Prl2a1*, and *Tjp1* (Applied Biosystems, Foster City, CA). Other primers (*18s*) and primer/probe mixes (*Stmn1* and *Ube2e1*) were designed with Primer Express software (Applied Biosystems) and were synthesized by IDT (Iowa City, IA). (Sequences were: *Stmn1* forward, TGGCCAGTGTCCTTTACTTTCCPCR; *Stmn1* probe, FAM-CTAGAAGCCGATGTAGGACCGT-TAMRA; *Stmn1* reverse, CCCTTGAGCCCCTA AAACATC; *Ube2e1* forward, CCAGCCC-TAACCATCTCGAA; *Ube2e1* probe, FAM-CTCC TTTCTATCTGCT-CACTCCTTAC-TAMRA; *Ube2e1* reverse, CCAAAGGGTCAGCAGGA TTG; *18s* forward, TTCGGAACTGAGGCCATGAT; and *18s* reverse, TTTCGCTCTGGTCCG TCTTG.) Reactions were carried out in triplicate with Taqman Universal PCR Master Mix or SYBR Green Master Mix (18s; Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR System.

All primer/probe sets were validated using serial dilutions of a pooled RNA sample. Results for each gene of interest were normalized to an *18s* RNA control.

Feulgen Staining

Mouse trophoblasts were isolated as described above and were plated on poly-L-lysine-coated and Matrigel-coated glass coverslips in six-well plates in medium containing 10% FBS. Cells were allowed to adhere by culturing overnight, and a set of (T_0 control) samples then was fixed in 4% paraformaldehyde for 15 min. The other coverslips were placed in serum-free medium for an additional 24 h with (leptin 24) or without (control 24) 50 ng/ml recombinant mouse leptin (Sigma) before fixation. Cells were stained using the Blue Feulgen DNA Ploidy Analysis Staining Kit (Scytek, Logan, UT) according to the manufacturer's instructions. Briefly, coverslips were placed in 5 M HCl for 30 min, rinsed, and then stained for 60 min, followed by three rinses in sodium bisulfite (5 min each), and three rinses in distilled H_2O. Specimens on coverslips were dehydrated and mounted, specimen side face down, on a glass slide. Ten different fields from each coverslip were imaged under white light under an Olympus IX70 microscope attached to a SenSys CCD camera. Images were acquired using MetaMorph version 6.3r6 software (Molecular Devices, Sunnyvale, CA). Metamorph was also used to outline each nucleus on the images and to calculate staining intensity and area. Three independent experiments were performed, and a total of 1373 (control 0), 1249 (control 24), and 1182 (leptin 24) experimental cells were analyzed. Mean nucleic acid content (staining intensity × area) was compared among groups by one-way ANOVA using GraphPad Prism. Nucleic acid content distributions of the control 24 and leptin 24 groups were compared by Komolgorov-Smirnov test using SAS.

RESULTS

STAT3 Signaling

Experiments were performed to determine whether control and leptin-treated trophoblast cells displayed evidence of STAT3 activation as indicated by increased phosphorylation at serine 727 and tyrosine 705 residues (Fig. 1, a and b). No increase in STAT3 phosphorylation at either residue was detected after cells had been treated with leptin for 3, 10, 30, or 60 min. Instead, there was a significant decrease in phosphorylation both at S727 and Y705 after leptin treatment for 1 h. Total STAT3 concentrations (i.e., phosphorylated and nonphosphorylated forms) remained unchanged across all time points examined (n = 3; data not shown). To explore a potential mechanism for the observed inhibition of tyrosine phosphorylation of STAT3, the relative concentrations of SOCS3

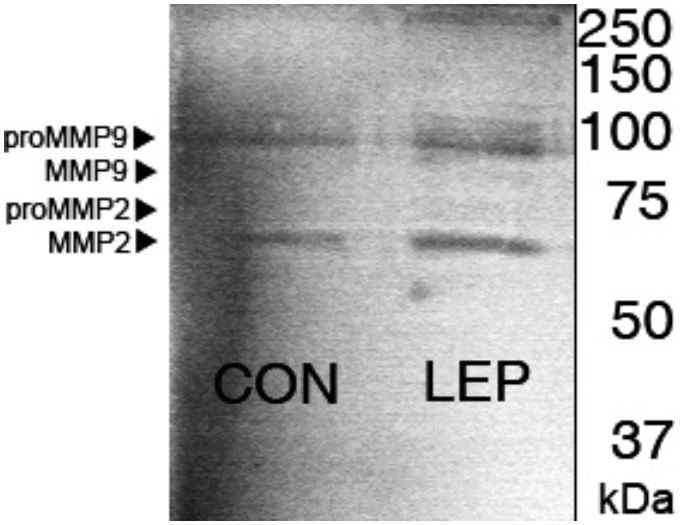

FIG. 2. Gelatin zymography of trophoblast cell culture medium. There are two major bands of gelatinocytic activity at approximately 92 kDa, the size of pro-matrix metalloproteinase 9 (proMMP9), and at approximately 65 kDa, the size of active matrix metalloproteinase 2 (MMP2). There are faint bands at 86 kDa, the size of active MMP9, and 72 kDa, the size of pro-MMP2 (proMMP2).

protein were measured and found to be significantly increased after 1 h of leptin treatment (Fig. 1c).

MEK Signaling

In contrast to its ability to inhibit STAT3 phosphorylation, leptin activated the MEK/ERK signaling pathway. MEK (MAP2K1) phosphorylation was increased after 3 min of leptin treatment but returned to basal levels after 1 h (Fig. 1d).

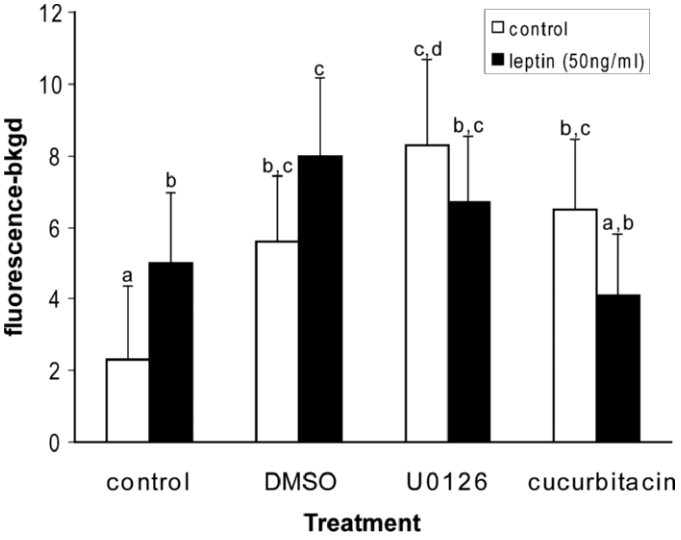

FIG. 3. Effect of MAP2K1 (MEK (U0126) inhibitor and STAT3 (cucurbitacin I) inhibitors on leptin-stimulated metalloproteinase activity. Dimethyl sulfoxide was the vehicle for both inhibitors. The y-axis shows activity of fluorescent gelatinase substrate in sample medium—fluorescence in medium not exposed to cells (bkgd). Metalloproteinase activity was significantly higher in the medium of primary trophoblast cells treated with 50 ng/ml leptin compared with cells cultured in serum-free medium alone. Cells treated with cucurbitacin and leptin had significantly less activity than those treated with leptin and vehicle (DMSO) only. Columns with different letters are significantly different (ANOVA, Tukey test $P < 0.05$). Bars represent standard error. N = 5.

TABLE 1. Numbers of genes with significantly different expression determined by microarray analysis using different statistical comparisons and significance levels.

Comparison	$P < 0.05$	$P < 0.01$	$P < 0.01$ (fold change >1.5)	False discovery rate 0.2
1 h control vs. 1 h leptin	998	149	0	0
1 h leptin vs. 6 h leptin	2059	669	122	206
24 h control vs. 24 h leptin	1465	117	42	0
(24 h–1 h leptin) vs. (24 h–1 h control)	1818	229	188	0

MMP Activity Assays

We next tested whether leptin stimulated metalloproteinase activity in mouse primary trophoblast cultures, as it does in human ones, and whether this activity was dependent on either STAT3 or MAP2K1 (MEK) signaling. Gelatin zymography revealed that a protein of approximately 92 kDa, the molecular mass of pro-matrix metalloproteinase 9 (pro-MMP9), was the most active gelatinase released by trophoblast cells (Fig. 2). A protein of approximately 65 kDa, corresponding to active MMP2, was also evident, although faint bands corresponding to active MMP9 (86 kDa) and pro-MMP2 (72 kDa) were also visible. Leptin appeared to stimulate the activity of both metalloproteinases.

Trophoblast cells were cultured with U0126, a MEK (MAP2K1) inhibitor, or cucurbitacin I, a STAT3 inhibitor, and the effect on metalloproteinase activity was measured using a fluorescent gelatinase substrate (Fig. 3). As expected, leptin significantly increased MMP activity. Dimethyl sulfoxide, the vehicle in which both inhibitors were delivered, had a similar effect. Together, DMSO and leptin had a roughly additive effect, which was significantly higher than that of leptin, but not DMSO, by itself. This additive effect was not observed when U0126 was also present in the medium. However, these treatment groups (DMSO, DMSO + leptin, and U0126 + leptin) were not statistically different. Cucurbitacin I had no effect in the absence of leptin, but significantly decreased activity versus that seen with DMSO + leptin.

Microarray Analysis

Illumina microarrays containing 24 886 probe sets were used to measure changes in gene expression as a result of leptin treatment in the mouse primary trophoblast cells, with the expectation that there would be an increase in expression of those genes normally associated with giant cells, the main invasive component of mouse placenta at this stage. Raw and normalized data are available in the GEO database, accession no. GSE10297 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi). To identify the cascade of events occurring over time, expression was analyzed after 1, 6, and 24 h of leptin treatment. Cells cultured in the absence of leptin were also examined at 1 and 24 h. Four statistical comparisons were made: pairwise comparisons between control and leptin-treated samples at 1 h (Supplemental Data, available online at www.biolreprod.org), the contrast between leptin treatments of 1 h and 6 h (Supplemental Data), the contrast between leptin-treated and control cells at 24 h, and a comparison of changes occurring over time in the leptin-treated samples versus changes over time in the control samples ([24 h − 1 h leptin] – [24 h − 1 h control]; Supplemental Data). The numbers of significantly

TABLE 2. The DAVID functional annotation clustering algorithim was used to identify gene function categories present among the lists of genes with significantly different gene expression.

	$P < 0.05$		$P < 0.01$	
Gene function category[a]	No. genes	Score[b]	No. genes	Score[b]
Control 1 h vs. leptin 1 h				
Cell cycle/mitosis	47	3.11	7	0.75
Extracellular matrix	21	2.25	5	1.58
Oxidoreductase/energy generation	59	1.95	8	0.3
Gap junction/cell communication	13	1.83	1	0
Endocytosis/vesicle transport	17	1.81	7	0.9
Tight junction/cell adhesion	33	1.63	4	1.1
TGFB signaling pathway	21	1.45	2	0
Ubiquitin/Zn finger RING-type	41	1.35	5	0.95
Polysaccharide binding	13	1.32	4	0.63
Inflammatory response	68	1.27	8	0.09
Leptin 1 h vs. leptin 6 h				
Cell cycle/mitosis	94	8.7	35	4.64
Development/differentiation	43	6.49	41	3.68
Apoptosis	79	4.87	34	3.4
Angiogenesis	58	3.47	23	2.52
Protease/peptidase/zymogen	84	2.37	35	1.34
Lipid transport	16	2.34	6	0.81
Cell proliferation	50	2.24	40	1.83
Cytoskeleton binding/ actin-binding	37	2.13	19	2.78
Cell motility	36	2.11	16	1.14
Extracellular matrix	29	1.23	13	1.53
(24 h–1 h Leptin) vs. (24 h–1 h control)				
Ubiquitin/ligase activity	90	4.58	16	1.02
Cell cycle/ mitosis	82	4.2	13	1.28
Ribosome biogenesis/ rrna processing	21	2.3	2	0
Translation initiation	26	1.98	3	0
Tricarboxylic acid cycle	10	1.87	0	0
Chaperone/protein folding	311	1.71	2	0
GTP binding/GTPase	56	1.56	10	0.64
Mitotic spindle/microtubule organization	10	1.33	14	1.07
Nonsense-mediated mRNA decay	5	1.17	1	0
Cell junctions/cell-matrix junctions	21	1.17	1	0

[a] The 10 categories with the highest scores for each comparison are shown, excluding redundant or overly general ("organelle," "cellular physiological process") categories.
[b] Higher scores indicate a higher likelihood that the category is significantly represented.

regulated genes for each comparison are shown in Table 1. The functional annotation clustering algorithm of DAVID [29] (http://david.abcc.ncifcrf.gov/) was used to identify regulated pathways and gene function categories. This program groups similar gene ontology categories to simplify annotation of microarray data. In addition, enrichment scores based on the Fisher exact test indicate whether a particular category is more represented than would be expected by chance alone. The higher the score, the more likely that the functional category is truly regulated and not represented by chance alone. More than 150 categories were identified for the lists of genes that were differentially expressed at statistically significant levels ($P < 0.05$) for each comparison. Some categories ("cellular processes," "DNA binding") are so broad as to be meaningless, whereas others overlap considerably, but with these excluded, categories receiving the 10 highest enrichment scores are listed in Table 2. This method was used to analyze the lists of genes that were differentially expressed at two different statistical significance levels (0.05 and 0.01) for each of the three statistical comparisons (1 h LEP vs. 1 h CON, 1 h LEP vs. 6 h LEP, 1–24 h LEP vs. 1–24 h CON). Enrichment scores are given for each list.

TABLE 3. Comparison of gene expression changes measured by microarray and real-time RT-PCR.

	Fold change (24 h leptin vs. 24 h control)		
Gene	PCR average	Range	Array
Mdh2	1.97	1.31–2.54	4.26
Prl2a1	2.15	1.05–3.53	1.85
Stmn1	4.67	1.30–6.41	3.86
Tjp1	1.99	0.81–4.18	2.43
Ube2e	1.50	0.84–2.72	2.43

In addition to the functional categories recognized by DAVID, some of the regulated genes are known to be involved in trophoblast differentiation and function. For example, leptin upregulated expression of prolactin-like protein m (*Prl2a1*), inhibitor of DNA binding 2 (*Id2*), and cathepsin q (*Ctsq*) over 24 h (Fig. 4a and Supplemental Data). The microarray also showed a decline over time in expression of *Map2k1* (MEK) and *Nras* (neuroblastoma RAS viral oncogene homolog), which is upstream of MEK, in control but not leptin-treated cells. Thus, at 24 h *Map2k1* and *Nras* were 3.6- and 1.8-fold higher, respectively, in leptin-treated cells than in controls.

Five genes were used for technical validation of the microarray results by real-time PCR (Table 3). Genes were chosen to represent different functional categories of biological interest from Table 2 (tricarboxylic acid cycle, trophoblast differentiation, cell cycle regulation, cell junctions, and ubiquitination). Within these functional categories, genes that showed statistically significantly different expression profile patterns over time with leptin treatment versus control (Supplemental Data) with the greatest fold change were used. These were *Mdh2* (malate dehydrogenase 2), *Prl2a1*, *Stmn1* (stathmin 1), *Tjp1* (tight junction protein 1/zona occludins), and *Ube2e1* (ubiquitin-conjugating enzyme E2E 1).

Real-time RT-PCR was used to compare expression in 24 h LEP and 24 h CON samples. The RT-PCR and microarray data were consistent, with all five genes showing higher expression in the leptin than the control 24 h samples, whichever method was used for comparison. In most cases, the fold changes were comparable, although *Mdh2* showed a greater fold change on the microarray than in the PCR (Table 3).

Feulgen Staining

The functional category that was most highly represented on the lists of changed genes at 1 and 6 h and was second at 24 h was cell cycle regulation (Table 2). The major invasive trophoblast subtype in the mouse placenta, the giant cell, undergoes multiple rounds of endoreduplication and becomes polyploid, with the proliferating giant cells of the junctional zone showing 2–8c, and the secondary giant cells eventually able to reach 512-1024c [30]. The array results show that in both the control and leptin-treated cells after 24 h of culture, there was increased expression of genes associated with polyploidy, such as *Ccnd1* (cyclin D1), *Cdkn2a* (cyclin-dependent kinase inhibitor 2A), and *Aurka* (aurora kinase a), and a decreased expression of genes, such as *Gmnn* (geminin), *Stmn1*, and *Ccna2* (cyclin A2), that suppress polyploidy (Fig. 4, b and c). However, these changes were greater in the control cells than in the leptin-treated cells, suggesting that leptin might actually inhibit rather than drive the process of endoreduplication and increased ploidy. Accordingly, the

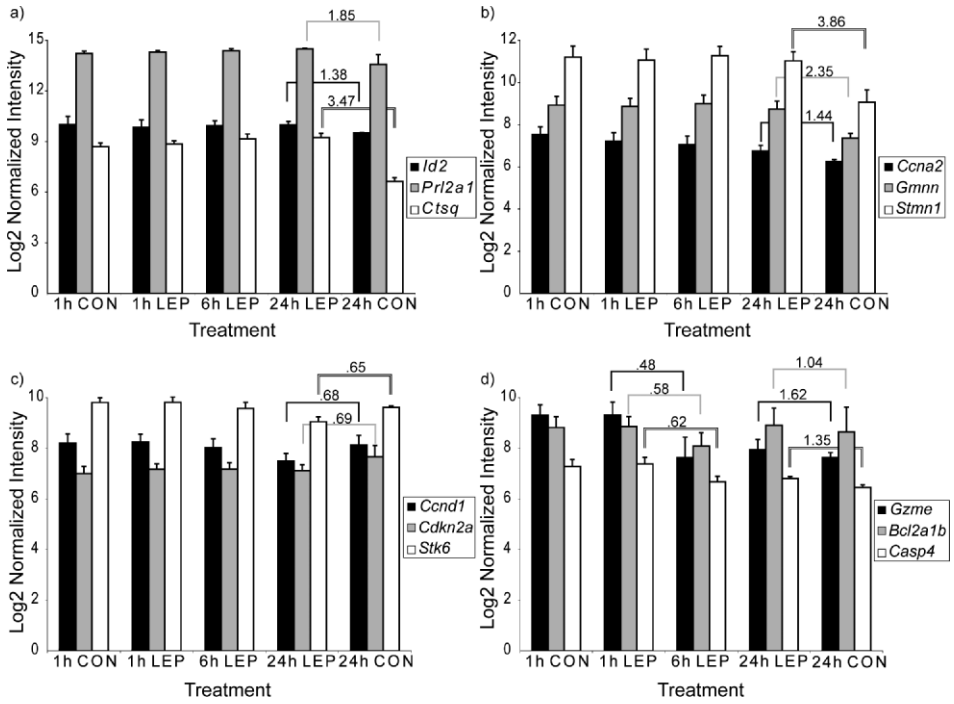

FIG. 4. Expression profiles of selected genes by microarray. Columns represent mean normalized expression levels on a log2 scale. Fold changes (LEP/CON) at 24 h on the raw scale are indicated above the columns. Bars represent standard error. **a**) Trophoblast-specific genes. **b**) Genes negatively associated with polyploidy. **c**) Genes positively associated with polyploidy. **d**) Apoptosis-related genes.

relative DNA content of individual cells was compared by Feulgen staining either just after plating cells on coverslips (0 h) or after 24 h in the presence or absence of leptin (Fig. 5a). As expected, cells with small nuclei and low DNA content, most likely giant cell precursors, appeared to be more numerous at time 0 than after 24 h of culture. However, it was also clear that even at the time of plating, the majority of cells already possessed the characteristic large nuclei of trophoblast giant cells (Fig. 5, a–c). This observation was supported by the microarray data, which showed extremely high levels of expression of *Prl3b1* (*Csh2*/placental lactogen 2), a trophoblast giant cell marker, at all time points under both treatment conditions (Supplemental Data). Unexpectedly, given the microarray data, mean nucleic acid content index at 24 h (assessed by nuclear staining intensity $\times$ area) was significantly higher in leptin-treated cells than in controls in three independent experiments (Fig. 5, d–f).

Data from the three experiments were also combined and plotted on a single frequency distribution graph (Fig. 5g). The two distributions at 24 h were significantly different (Komolgorov-Smirnov test, $P < 0.0001$). Visual examination of the distributions reveals there was no dramatic difference in the number of cells to the right of the peak; that is, those cells with a higher-than-median ploidy. However, there was a clear difference to the left of the peak; that is, control cultures had more cells with low DNA content than did leptin-treated cultures. For example, 23.2% of the control cells had a nucleic acid content index lower than 300 000, whereas only 11.6% of leptin-treated cells fell in this range. Therefore, the increase in average DNA content following leptin treatment resulted largely from a loss of cells with low ploidy rather than a gain of cells with high ploidy.

DISCUSSION

As in a previous study in choriocarcinoma cells, leptin treatment did not activate STAT3 phosphorylation in primary mouse trophoblast cells [17]. However, phosphorylated STAT3 was detectable in the treated and untreated cells, a result that contrasts with findings in BeWo cells, which displayed no phosphorylated STAT3 either before or after leptin treatment [17]. Although leptin treatment did not alter the status of STAT3 phosphorylation, it did increase the expression of SOCS3. The rapid increase in SOCS3 protein (within 1 h) might be due to an increase in the stability of the protein, as has been demonstrated for SOCS3 in other systems [31]. However, SOCS3 mRNA induction has been detected in response to leptin in as little as 30 min in rat INS-1 insulinoma cells [32]. Therefore, the increase in SOCS3 could be an outcome of transcriptional upregulation of its gene, increased mRNA stability, reduced protein turnover, or a combination of these three factors. SOCS3 has been identified as a negative feedback regulator of activated STAT3 signaling by the leptin receptor and functions to inhibit JAK2 activation [33]. Our finding of SOCS3 activation in the absence of any change in STAT3 phosphorylation status suggests that SOCS3 is acting in an independent signaling capacity and not as part of a STAT3 feedback loop. A somewhat similar effect occurs in pancreatic beta cells, in which leptin treatment induces both STAT3 and SOCS3 activity. However, the net effect of leptin treatment on gene expression reflected the direct action of SOCS3 rather than its ability to antagonize STAT3 [32].

SOCS3 induction is generally considered to occur as a direct outcome of STAT3 phosphorylation, as has been observed in pancreatic beta cells [32] and some other systems [34, 35]. In the RCHO-1 immortalized rat trophoblast cell line, expression of SOCS3 is both STAT3 and MAP2K1 (MEK) dependent [36]. Because both are present but only MEK is being activated by leptin treatment, it is likely that constitutively phosphorylated STAT3 and leptin-stimulated MAP2K1 (MEK) action are driving the increased SOCS3 expression in our experiments.

Our finding that MAP2K1 (MEK) shows an immediate increase in phosphorylation after leptin treatment of trophoblast cells is consistent with the activation of MAPK3/MAPK1 (ERK 1/2) by leptin in BeWo and JAr choriocarcinoma cells [17, 18]. Thus, even though leptin stimulates proliferation in choriocarcinoma cells and appears not to do so in primary trophoblasts, there is an apparent conservation of this cell signaling mechanism [17–20]. As in primary human tropho-

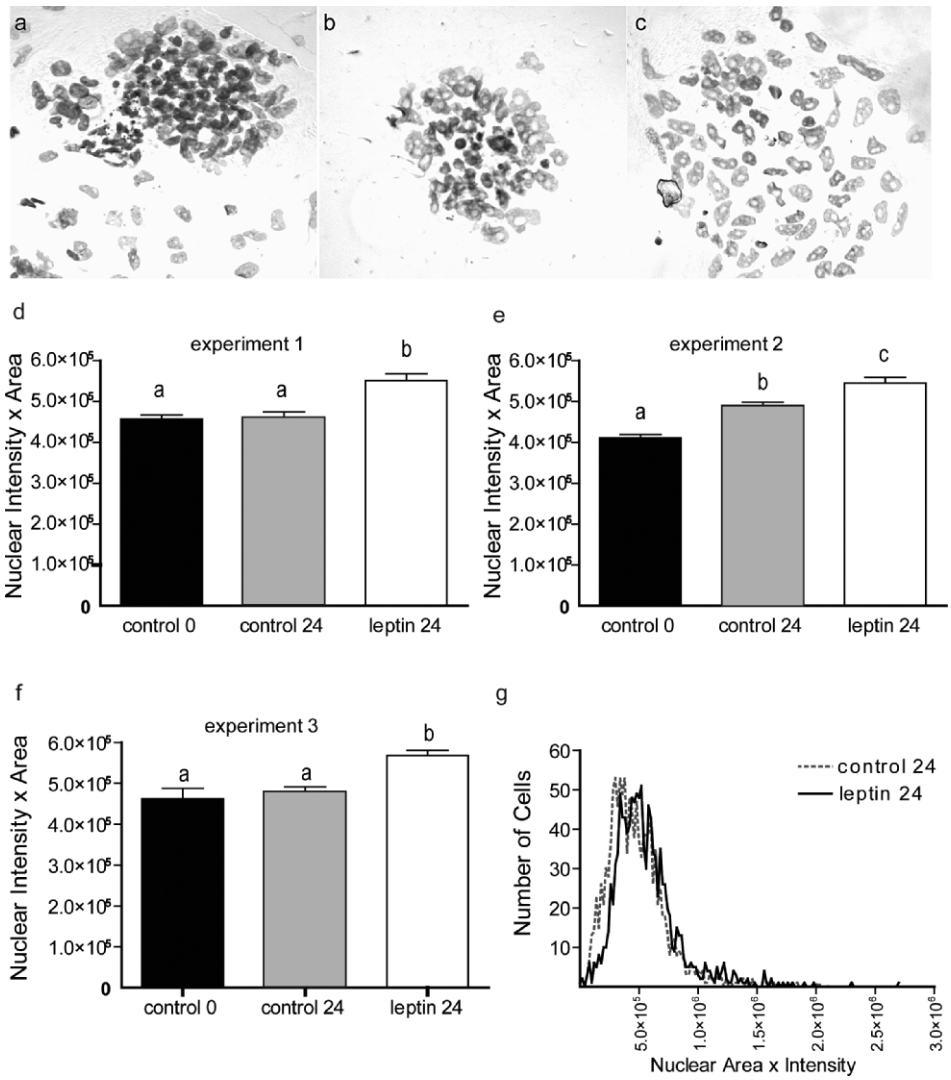

FIG. 5. Feulgen staining of primary trophoblast cells after plating (control 0) and 24 h later with (leptin 24) or without (control 24) leptin treatment. Representative photographs of control 0 (**a**), control 24 (**b**), and leptin 24 (**c**). Original magnification ×200. **d**–**f**) Average nucleic acid content of cultured trophoblast cells in three independent experiments, determined by multiplying Feulgen stain intensity × area in arbitrary units. **g**) Frequency distribution showing the number of cells with each nucleic acid content in the control 24 and leptin 24 groups across all three experiments.

blast cultures [10], leptin promoted a modest increase in MMP activity in the mouse cells, which likely contributes to their increased invasive potential. In the presence of the MAP2K1 (MEK) inhibitor U0126, leptin was unable to stimulate MMP activity. However, the stimulatory effect of the inhibitor vehicle, DMSO, masked much of the leptin effect. Thus, although the inhibitor data are consistent with a role for MAP2K1 (MEK) in mediating leptin-stimulated MMP activity, we are unable to reach an unequivocal conclusion.

STAT3 is highly expressed in cultured human trophoblasts retrieved early in pregnancy, when extravillous trophoblast cells are highly invasive. Inhibition of STAT3 in such cells is effective at reducing invasiveness [37, 38]. In mice, differentiation of invasive giant cells is regulated by the balance between LIF signaling via STAT3 and SOCS3 [39, 40]. Although the overall effect of leptin is to promote trophoblast invasion in vitro [19, 20], in the present study it inhibited STAT3. Indeed, the STAT3 inhibitor cucurbitacin I significantly inhibited MMP activity only in the presence of leptin, consistent with a further suppression of STAT3 activity. Thus, leptin may have a mixed effect on trophoblast invasion, with simultaneous activation of MAP2K1 (MEK) signaling and inhibition of STAT3 signaling. It will be interesting to determine the net effect of leptin in the presence of factors such as LIF that activate trophoblast invasion via the STAT3 pathway [14].

The microarray data provide general support for the conclusion that MAP2K1 (MEK) signaling is being influenced by leptin treatment. For example, the "MAPK cascade" was identified as a significantly represented functional category in all three gene lists in the DAVID analysis, although it was not among the highest-scoring categories, which are listed in Table 2. The gene for MEK itself (*Map2k1*) was expressed significantly higher at 24 h in the leptin-treated versus control samples.

Not surprisingly, the MEK pathway was only one of many signaling pathways affected by leptin treatment and/or by time in culture. There were changes in gene expression of several members of the RhoGTPase family in all three comparisons, including increases over time in expression of *Rhoc* and *Rnd3* (RhoE) in leptin-treated but not control cells. In addition, extremely small (less than 1.5-fold) but statistically significant differences in *Rhoa* (higher in leptin) and *Rock2* (higher in control) were seen at 1 h, suggesting dynamic regulation of this pathway by leptin, but not a specific direction of change. Both RHOA and RHOC are involved in control of focal adhesion and cell motility in many cell types, including human extravillous trophoblasts [41]. In addition, both MAP2K1 (MEK) and rho-dependent kinase activity have been implicated in leptin stimulation of invasion in cancer cells [42]. In pancreatic tumor cells, RHOC promotes invasion following loss of caveolin 1 [43]. The observation that the gene for

caveolin 1 (*Cav1*) is significantly downregulated at 6 h in our experiments is consistent with a role for RHOC in directing leptin-stimulated invasion of mouse trophoblast cells.

A comparison of gene expression profiles at 1 h and 6 h after leptin addition to the cultures indicates a general downregulation of both proapoptotic and antiapoptotic genes. For example, there is lowered expression of antiapoptotic Bcl2-related genes (*Bcl2*, *Bcl2l1*, *Bcl2l2*, *Bcl2a1a*, *Bcl2a1b*, and *Bcl2a1d*), as well as proapoptotic granzymes (*Gzm d*, *e*, *f*, and *g*) and caspases (*Casp 4*, *8*), but these changes may be unrelated to the action of leptin. Rather, they are probably correlated with cell death occurring during the first hours of culture, because by 24 h, differences in expression of the majority of apoptotic genes between leptin and control cultures were not evident (Fig. 4d and Supplemental Data), and the apoptosis pathway was not on the list of significantly enriched functional categories for genes regulated significantly by leptin over 24 h (Table 2). Interestingly, some of the granzyme genes (*Gzm d*, *e*, *f*, and *g*) and *Casp4* had significantly higher expression in the leptin-treated cells at 24 h. We are unable to explain these late changes, although it is possible that the granzymes are serving a function unrelated to apoptosis, such as in digesting extracellular matrix components [44].

The primary goal of this paper was to identify intracellular mechanisms whereby leptin increases trophoblast invasion. Accordingly, among the functional categories of genes regulated by leptin on the microarrays, "cell motility" (enrichment score 0.37; 19 genes for time-leptin interaction) is of particular interest. The most upregulated gene in this grouping was *Stmn1*, whose changed expression was confirmed by real-time PCR analysis. Its product, stathmin, has been identified as a key effecter of cell migration in *Drosophila* border cells, a model of normal physiological (nonmetastatic) cell migration [45]. Stathmin also promotes invasiveness of sarcoma cells [46] and motility of cultured gonadotropin-releasing hormone neurons [47]. The protein is a well-established regulator of microtubule dynamics during mitotic spindle formation, and indeed it was also classified in the "cell cycle" and "mitotic spindle" categories by the cluster analysis program. Its role as a regulator of microtubule dynamics is likely responsible for its involvement in the category "control of cell motility" [48]. Thus, stathmin is a good candidate for further investigations of mechanisms of trophoblast invasion, and particularly of how leptin stimulates this and related processes in other cell types.

Stathmin may play a second role in invading trophoblast cells; namely, in controlling endoduplication, because stathmin downregulation is required for genome duplication and endopolyploidy in megakaryocytes [49]. Similarly, geminin (*Gmnn*), whose expression was maintained by the presence of leptin over 24 h, is repressed during megakaryocyte differentiation [50]. Moreover, deletion *of Gmnn* induces endoreduplication and premature differentiation of giant cells in the early mouse embryo [51]. In contrast, *Aurka* (aurora kinase a), which was downregulated by leptin over 24 h, is positively associated with polyploidy [52, 53]. Similarly, MacAuley et al. [54] reported a switch from cyclin D3 to D1 with the onset of endoreduplication in mouse giant cells, whereas we observed a decrease in *Ccnd1* expression with leptin treatment relative to controls at 24 h. Together, these data are best explained if leptin treatment is inhibiting rather than promoting genome endoreduplication in these primary trophoblast cells. At first glance, however, the Feulgen data appear to contradict this finding. Figure 5 indicates that the leptin-treated cells had a higher average DNA content than the control groups. On the other hand, careful examination of the frequency distribution of cells over the range of DNA contents revealed a more subtle effect. After leptin treatment there was a shift in the curve from cells of low DNA content to ones with a midrange content, but little change in the number of cells with the highest amounts of DNA. In other words, there may be no increase in the number of terminally differentiated giant cells, merely a shift toward cells of intermediary phenotype.

Parast et al. [55] observed three stages of giant cell differentiation in Rcho-1 and primary rat trophoblast cultures. In the earliest stage, cells were nonmotile and proliferative. In the second, cells were highly motile but not dividing. In the third, terminally differentiated stage, cells were nonmotile and nonproliferative, with well-developed cell junctions. A similar phenomenon likely occurs in mouse trophoblast cells. Our data suggest that leptin is driving stage 1 cells to stage 2 and perhaps inhibiting the transition to stage 3. In particular, the Feulgen staining indicates a loss of cells at the earliest stage of differentiation and no gain in stage 3, whereas the array data are consistent with the view that leptin slows terminal differentiation. One possible explanation for the above results is that leptin somehow modulates transforming growth factor beta (TGFB) signaling, which has been implicated in driving terminal differentiation of trophoblasts of Rcho cells [56, 57] and inhibiting invasion of primary and immortalized human trophoblast cells [58, 59]. That leptin might promote early differentiation events but inhibit advancement to a terminally differentiated state is supported by the observation that genes associated with the TGFB signaling pathway are initially upregulated by leptin (Table 2) but subsequently show decreased expression. In particular, transcript concentrations for TGFBR2 and SMAD3 decline by 24 h, whereas those representing the inhibitor decorin are increased relative to controls.

It will be important to determine how these in vitro differentiation stages correlate with in vivo differentiation events and with the subpopulations of trophoblast giant cells that are present in the mature mouse placenta. Giant cells, as defined by their elevated ploidy, are found in multiple locations in the placenta and may have quite different phenotypes and functions [60]. To better understand how leptin may be affecting the differentiation of precursor cells to these subtypes, we examined regulation of genes identified as being preferentially expressed in one or more subpopulations of giant cells in vivo [60–63]. At 24 h, leptin upregulated genes said to specifically mark several different subtypes: *Ctsq* (cathepsin q), *Prl3d1* (prolactin family 3, subfamily d, member 1, or placental lactogen 1), *Prl2a1*, and *Prl7d1* (prolactin family 7, subfamily d, member 1, or proliferin-related protein; Fig. 4 and Supplemental Data). Thus, there is no evidence that leptin drives differentiation of any one of these in vivo subtypes at the expense of the others, or that any of these marker subtypes individually represents the "stage 2" differentiation that we see in vitro.

Overall, our data indicate that leptin acts through several signal transduction intermediates, including MAP2K1 (MEK) and SOCS3, to influence the differentiation of primary trophoblast cells. We suggest that leptin accelerates disappearance of non-giant cells while inhibiting terminal differentiation of committed giant cells. Leptin's ability to maintain trophoblast cells at an intermediary stage of differentiation likely contributes to their increased invasiveness relative to controls.

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REFERENCES

1. Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. Science 1994; 266:1508–1518.
2. Malik NM, Carter ND, Murray JF, Scaramuzzi RJ, Wilson CA, Stock MJ. Leptin requirement for conception, implantation, and gestation in the mouse. Endocrinology 2001; 142:5198–5202.
3. Ramos MP, Rueda BR, Leavis PC, Gonzalez RR. Leptin serves as an upstream activator of an obligatory signaling cascade in the embryo-implantation process. Endocrinology 2005; 146:694–701.
4. Hoggard N, Hunter L, Duncan JS, Williams LM, Trayhurn P, Mercer JG. Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta. Proc Natl Acad Sci U S A 1997; 94:11073–11078.
5. Zhao J, Townsend KL, Schulz LC, Kunz TH, Li C, Widmaier EP. Leptin receptor expression increases in placenta, but not hypothalamus, during gestation in *Mus musculus* and *Myotis lucifugus*. Placenta 2004; 25:712–722.
6. Challier J, Galtier M, Bintein T, Cortez A, Lepercq J, Hauguel-de Mouzon S. Placental leptin receptor isoforms in normal and pathological pregnancies. Placenta 2003; 24:92–99.
7. Smith JT, Waddell BJ. Leptin receptor expression in the rat placenta: changes in ob-ra, ob-rb, and ob-re with gestational age and suppression by glucocorticoids. Biol Reprod 2002; 67:1204–1210.
8. Islami D, Bischof P, Chardonnens D. Possible interactions between leptin, gonadotrophin-releasing hormone (GnRH-I and II) and human chorionic gonadotrophin (hCG). Eur J Obstet Gynecol Reprod Biol 2003; 110:169–175.
9. Cameo P, Bischof P, Calvo JC. Effect of leptin on progesterone, human chorionic gonadotropin, and interleukin-6 secretion by human term trophoblast cells in culture. Biol Reprod 2003; 68:472–477.
10. Castellucci M, De Matteis R, Meisser A, Cancello R, Monsurro V, Islami D, Sarzani R, Marzioni D, Cinti S, Bischof P. Leptin modulates extracellular matrix molecules and metalloproteinases: possible implications for trophoblast invasion. Mol Hum Reprod 2000; 6:951–958.
11. Ghilardi N, Uotani S, Wiestner A, Flier JS. Divergent signaling by the leptin receptor. J Biol Chem 1996; 93:6231–6235.
12. Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, Wang Y, Banks AS, Lavery HJ, Haq AK, Maratos-Flier E, Neel BG, Schwartz MW, et al. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature 2003; 421:856–859.
13. Fitzgerald JS, Busch S, Wengenmayer T, Foerster K, de la Motte T, Poehlmann TG, Markert UR. Signal transduction in trophoblast invasion. Chem Immunol Allergy 2005; 88:181–199.
14. Fitzgerald JS, Tsareva SA, Poehlmann TG, Berod L, Meissner A, Corvinus FM, Wiederanders B, Pfitzner E, Markert UR, Friedrich K. Leukemia inhibitory factor triggers activation of signal transducer and activator of transcription 3, proliferation, invasiveness, and altered protease expression in choriocarcinoma cells. Int J Biochem Cell Biol 2005; 37:2284–2296.
15. Qiu Q, Yang M, Tsang BK, Gruslin A. Both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling are required in epidermal growth factor-induced human trophoblast migration. Mol Hum Reprod 2004; 10:677–684.
16. Gleeson LM, Chakraborty C, McKinnon T, Lala PK. Insulin-like growth factor-binding protein 1 stimulates human trophoblast migration by signaling through alpha 5 beta 1 integrin via mitogen-activated protein Kinase pathway. J Clin Endocrinol Metab 2001; 86:2484–2493.
17. Cauzac M, Czuba D, Girard J, Hauguel-de Mouzon S. Transduction of leptin growth signals in placental cells is independent of JAK-STAT activation. Placenta 2003; 24:378–384.
18. Bifulco G, Trencia A, Caruso M, Tommaselli GA, Miele C, di Carlo C, Beguinot F, Nappi C. Leptin induces mitogenic effect on human choriocarcinoma cell line (JAr) via MAP kinase activation in a glucose-dependent fashion. Placenta 2003; 24:385–391.
19. Schulz LC, Townsend K, Kunz TH, Widmaier EP. Inhibition of trophoblast invasiveness in vitro by immunoneutralization of leptin in the bat, *Myotis lucifugus* (Chiroptera). Gen Comp Endocrinol 2007; 150:59–65.
20. Schulz LC, Widmaier EP. The effect of leptin on mouse trophoblast cell invasion. Biol Reprod 2004; 71:1963–1967.
21. Thordarson G, Folger P, Talamantes F. Development of a placental cell culture system for studying the control of mouse placental lactogen II secretion. Placenta 1987; 8:573–585.
22. Kronfeld-Schor N, Zhao J, Silvia BA, Bicer E, Mathews PT, Urban R, Zimmerman S, Kunz TH, Widmaier EP. Steroid-dependent up-regulation of adipose leptin secretion in vitro during pregnancy in mice. Biol Reprod 2000; 63:274–280.
23. Tomimatsu T, Yamaguchi M, Murakami T, Ogura K, Sakata M, Mitsuda N, Kanzaki T, Kurachi H, Irahara M, Miyake A, Shima K, Aono T, et al. Increase of mouse leptin production by adipose tissue after midpregnancy: gestational profile of serum leptin concentration. Biochem Biophys Res Commun 1997; 240:213–215.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
25. Ricke WA, Smith GW, Reynolds LP, Redmer DA, Smith MF. Matrix metalloproteinase (2, 9, and 14) expression, localization, and activity in ovine corpora lutea throughout the estrous cycle. Biol Reprod 2002; 66: 1083–1094.
26. Tabe Y, Konopleva M, Munsell MF, Marini FC, Zompetta C, McQueen T, Tsao T, Zhao S, Pierce S, Igari J, Estey EH, Andreeff M. PML-RARalpha is associated with leptin-receptor induction: the role of mesenchymal stem cell-derived adipocytes in APL cell survival. Blood 2004; 103:1815–1822.
27. Dunning MJ, Smith ML, Ritchie ME, Tavare S. beadarray: R classes and methods for Illumina bead-based data. Bioinformatics 2007; 23:2183–2184.
28. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004; 3:Article 3.
29. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 2003; 4:P3.
30. Zybina TG, Zybina EV. Cell reproduction and genome multiplication in the proliferative and invasive trophoblast cell populations of mammalian placenta. Cell Biol Int 2005; 29:1071–1083.
31. Sasaki A, Inagaki-Ohara K, Yoshida T, Yamanaka A, Sasaki M, Yasukawa H, Koromilas AE, Yoshimura A. The N-terminal truncated isoform of SOCS3 translated from an alternative initiation AUG codon under stress conditions is stable due to the lack of a major ubiquitination site, Lys-6. J Biol Chem 2003; 278:2432–2436.
32. Laubner K, Kieffer TJ, Lam NT, Niu X, Jakob F, Seufert J. Inhibition of preproinsulin gene expression by leptin induction of suppressor of cytokine signaling 3 in pancreatic beta-cells. Diabetes 2005; 54:3410–3417.
33. Bjorbaek C, Elmquist JK, El-Haschimi K, Kelly J, Ahima RS, Hileman S, Flier JS. Activation of SOCS-3 messenger ribonucleic acid in the hypothalamus by ciliary neurotrophic factor. Endocrinology 1999; 140: 2035–2043.
34. Auernhammer CJ, Bousquet C, Melmed S. Autoregulation of pituitary corticotroph SOCS-3 expression: characterization of the murine SOCS-3 promoter. Proc Natl Acad Sci U S A 1999; 96:6964–6969.
35. Banks AS, Davis SM, Bates SH, Myers MG Jr. Activation of downstream signals by the long form of the leptin receptor. J Biol Chem 2000; 275: 14563–14572.
36. Isobe A, Takeda T, Sakata M, Yamamoto T, Minekawa R, Hayashi M, Auernhammer CJ, Tasaka K, Murata Y. STAT3-mediated constitutive expression of SOCS3 in an undifferentiated rat trophoblast-like cell line. Placenta 2006; 27:912–918.
37. Poehlmann TG, Fitzgerald JS, Meissner A, Wengenmayer T, Schleussner E, Friedrich K, Markert UR. Trophoblast invasion: tuning through LIF, signalling via Stat3. Placenta 2005; 26(suppl A):S37–S41.
38. Corvinus FM, Fitzgerald JS, Friedrich K, Markert UR. Evidence for a correlation between trophoblast invasiveness and STAT3 activity. Am J Reprod Immunol 2003; 50:316–321.
39. Takahashi Y, Takahashi M, Carpino N, Jou ST, Chao JR, Tanaka S, Shigeyoshi Y, Parganas E, Ihle JN. Leukemia inhibitory factor regulates trophoblast giant cell differentiation via Janus kinase 1-signal transducer and activator of transcription 3-suppressor of cytokine signaling 3 pathway. Mol Endocrinol 2008; 22:1673–1681.
40. Takahashi Y, Carpino N, Cross JC, Torres M, Parganas E, Ihle JN. SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. EMBO J 2003; 22:372–384.
41. Shields SK, Nicola C, Chakraborty C. Rho guanosine 5′-triphosphatases differentially regulate insulin-like growth factor I (IGF-I) receptor-dependent and -independent actions of IGF-II on human trophoblast migration. Endocrinology 2007; 148:4906–4917.
42. Horiguchi A, Sumitomo M, Asakuma J, Asano T, Zheng R, Nanus DM, Hayakawa M. Leptin promotes invasiveness of murine renal cancer cells via extracellular signal-regulated kinases and rho dependent pathway. J Urol 2006; 176:1636–1641.
43. Lin M, DiVito MM, Merajver SD, Boyanapalli M, van Golen KL. Regulation of pancreatic cancer cell migration and invasion by RhoC GTPase and caveolin-1. Mol Cancer 2005; 4:21.

44. Buzza MS, Bird PI. Extracellular granzymes: current perspectives. Biol Chem 2006; 387:827–837.
45. Borghese L, Fletcher G, Mathieu J, Atzberger A, Eades WC, Cagan RL, Rorth P. Systematic analysis of the transcriptional switch inducing migration of border cells. Dev Cell 2006; 10:497–508.
46. Baldassarre G, Belletti B, Nicoloso MS, Schiappacassi M, Vecchione A, Spessotto P, Morrione A, Canzonieri V, Colombatti A. p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion. Cancer Cell 2005; 7:51–63.
47. Giampietro C, Luzzati F, Gambarotta G, Giacobini P, Boda E, Fasolo A, Perroteau I. Stathmin expression modulates migratory properties of GN-11 neurons in vitro. Endocrinology 2005; 146:1825–1834.
48. Niethammer P, Bastiaens P, Karsenti E. Stathmin-tubulin interaction gradients in motile and mitotic cells. Science 2004; 303:1862–1866.
49. Iancu-Rubin C, Nasrallah CA, Atweh GF. Stathmin prevents the transition from a normal to an endomitotic cell cycle during megakaryocytic differentiation. Cell Cycle 2005; 4:1774–1782.
50. Bermejo R, Vilaboa N, Cales C. Regulation of CDC6, geminin, and CDT1 in human cells that undergo polyploidization. Mol Biol Cell 2002; 13: 3989–4000.
51. Gonzalez MA, Tachibana KE, Adams DJ, van der Weyden L, Hemberger M, Coleman N, Bradley A, Laskey RA. Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development. Genes Dev 2006; 20:1880–1884.
52. Anand S, Penrhyn-Lowe S, Venkitaraman AR. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. Cancer Cell 2003; 3:51–62.
53. Huang X, Ruan Q, Fang Y, Traganos F, Darzynkiewicz Z, Dai W. Physical and functional interactions between mitotic kinases during polyploidization and megakaryocytic differentiation. Cell Cycle 2004; 3: 946–951.
54. MacAuley A, Cross JC, Werb Z. Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells. Mol Biol Cell 1998; 9:795–807.
55. Parast MM, Aeder S, Sutherland AE. Trophoblast giant-cell differentiation involves changes in cytoskeleton and cell motility. Dev Biol 2001; 230: 43–60.
56. Hamlin GP, Soares MJ. Regulation of deoxyribonucleic acid synthesis in proliferating and differentiating trophoblast cells: involvement of transferrin, transforming growth factor-beta, and tyrosine kinases. Endocrinology 1995; 136:322–331.
57. Rama S, Suresh Y, Rao AJ. TGF beta1 induces multiple independent signals to regulate human trophoblastic differentiation: mechanistic insights. Mol Cell Endocrinol 2003; 206:123–136.
58. Tse WK, Whitley GS, Cartwright JE. Transforming growth factor-beta1 regulates hepatocyte growth factor-induced trophoblast motility and invasion. Placenta 2002; 23:699–705.
59. Graham CH, Lala PK. Mechanism of control of trophoblast invasion in situ. J Cell Physiol 1991; 148:228–234.
60. Simmons DG, Fortier AL, Cross JC. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. Dev Biol 2007; 304:567–578.
61. Toft DJ, Linzer DI. Identification of three prolactin-related hormones as markers of invasive trophoblasts in the rat. Biol Reprod 2000; 63:519–525.
62. Ain R, Canham LN, Soares MJ. Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. Dev Biol 2003; 260:176–190.
63. Sahgal N, Knipp GT, Liu B, Chapman BM, Dai G, Soares MJ. Identification of two new nonclassical members of the rat prolactin family. J Mol Endocrinol 2000; 24:95–108.